JOURNAL OF VIROLOGY, May 1984, p. 507–514
0022-538X/84/050507-08$02.00/0

Vol. 50, No. 2

In Vitro Translation of Poliovirus RNA: Utilization of Internal Initiation Sites in Reticulocyte Lysate

ANDREW J. DORNER,[1]† BERT L. SEMLER,[1]‡ RICHARD J. JACKSON,[2] RONNIE HANECAK,[1]§ ELIZABETH DUPREY,[1] AND ECKARD WIMMER[1]*

Department of Microbiology, State University of New York at Stony Brook, Stony Brook, New York 11794,[1] and Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom[2]

Received 21 October 1983/Accepted 8 February 1984

The translation of poliovirus RNA in rabbit reticulocyte lysate was examined. Translation of poliovirus RNA in this cell-free system resulted in an electrophoretic profile of poliovirus-specific proteins distinct from that observed in vivo or after translation in poliovirus-infected HeLa cell extract. A group of proteins derived from the P3 region of the polyprotein was identified by immunoprecipitation, time course, and *N*-formyl-[^{35}S]methionine labeling studies to be the product of the initiation of protein synthesis at an internal site(s) located within the 3′-proximal RNA sequences. Utilization of this internal initiation site(s) on poliovirus RNA was abolished when reticulocyte lysate was supplemented with poliovirus-infected HeLa cell extract. Authentic P1-1a was also synthesized in reticulocyte lysate, indicating that correct 5′-proximal initiation of translation occurs in that system. We conclude that the deficiency of a component(s) of the reticulocyte lysate necessary for 5′-proximal initiation of poliovirus protein synthesis resulted in the ability of ribosomes to initiate translation on internal sequences. This aberrant initiation could be corrected by factors present in the HeLa cell extract. Apparently, under certain conditions, ribosomes are capable of recognizing internal sequences as authentic initiation sites.

For several decades, poliovirus-specific protein synthesis has been the subject of extensive investigation. Early studies of protein synthesis in poliovirus-infected HeLa cells resulted in the conclusion that translation of poliovirus RNA is initiated at a single site near the 5′ end of the RNA, resulting in the synthesis of a precursor polyprotein (NCVPOO) from which most, if not all, poliovirus-specific proteins are derived by proteolytic processing. In these studies of poliovirus protein synthesis in vivo, a protein with a molecular weight of 220,000 (that corresponded to the polyprotein) was identified in infected cells treated with amino acid analogs to inhibit protein processing (15, 16). The relative order on the genome of the coding sequences for the proteins derived from the polyprotein was determined with pactamycin, an inhibitor of the initiation of protein synthesis (24, 29, 30). Recent RNA and protein sequence analyses have confirmed the genetic map of poliovirus and identified the initiation site of synthesis of the poliovirus polyprotein (5, 10, 11, 17, 22, 25, 26). This work confirmed that the RNA is functionally monocistronic yet encodes a number of stable proteins that are the end products of a pathway of multiple proteolytic cleavages. On the basis of the major primary cleavages, the polyprotein has been divided into three regions (17): P1 includes the capsid proteins; P2 includes polypeptides P2-3b, P2-5b, and P2-X of unknown function; and P3 includes replication proteins such as VPg, the proteinase P3-7c, and the RNA polymerase P3-4b (Fig. 1).

Studies of poliovirus protein synthesis in vitro have proven difficult, owing to the inefficiency of translation of poliovirion RNA in cell-free protein synthesizing systems. However, beginning with an analysis of the interaction of poliovirion RNA with *Escherichia coli* ribosomes (33), a considerable body of literature has developed concerning the complexities of the translation of the poliovirus genome. Early in vitro work was hampered by premature termination of protein synthesis and aberrant processing of the protein products. However, several cell-free systems have been reported to translate the entire coding sequence of the RNA and to allow the processing of authentic poliovirus-specific proteins. Poliovirion RNA is an inefficient messenger in both the micrococcal nuclease-treated reticulocyte lysate (28) and in an extract of poliovirus-infected HeLa cells (32). Identification of the in vitro translation products by electrophoretic comparison with poliovirus-specific proteins found in virus-infected HeLa cells or by tryptic peptide analysis has shown that faithful translation and protein processing occur to a great degree in these systems (28, 32).

In *N*-formyl-[^{35}S]methionine labeling studies, including tryptic peptide analysis, two proteins synthesized from independent initiation events have been identified in both the reticulocyte lysate and the poliovirus-infected HeLa cell extract programmed with poliovirus RNA (3, 12). In these studies, utilization of the two different initiation sites on the RNA was modulated by the concentration of magnesium. At a low magnesium concentration (1.5 mM), a protein corresponding in size to P1-1a was labeled with *N*-formyl-[^{35}S]methionine, suggesting that P1-1a is an initiation protein. P1-1a has been mapped to the amino terminus of the polyprotein NCVPOO. This polyprotein is the only primary product of the initiation of protein synthesis found in vivo (24, 29, 30), and the encoding sequences for P1-1a have been determined to immediately follow the initiation codon for polyprotein synthesis (5). At a high magnesium concentration (4 mM), however, a small protein (5,000 to 10,000 in molecular weight) was predominantly labeled with *N*-formyl-[^{35}S]methionine in vitro. It does not share an amino-terminal tryptic peptide with P1-1a and so is not the product

* Corresponding author.
† Present address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA 02111.
‡ Present address: Department of Microbiology, College of Medicine, University of California, Irvine, CA 92717.
§ Present address: Department of Molecular Biology and Biochemistry, University of California, Irvine, CA 92717.

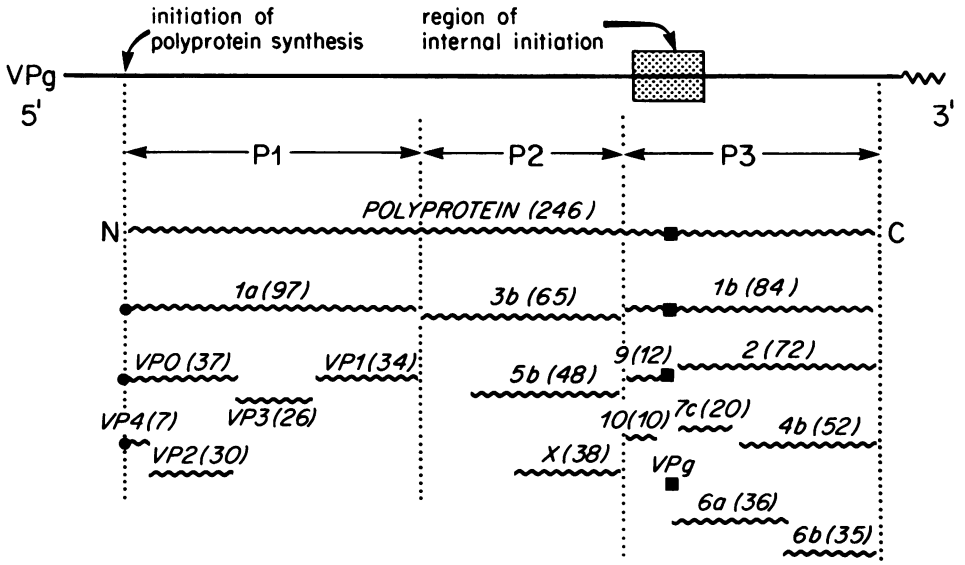

FIG. 1. Genomic organization of poliovirus (17). The solid line represents the genome RNA, which has a 5'-terminal covalently linked protein, VPg, and 3'-terminal polyadenylate tract. The wavy lines represent the virus-encoded polypeptides. The polyprotein has been divided into three regions, P1, P2, and P3, that are used as a prefix to identify the products of proteolytic processing (17). The numbers in parentheses are the molecular weights ($\times 10^{-3}$) calculated from the predicted amino acid sequence (17). Closed circles on P1-1a. VP0, and VP4 indicate the presence of blocked amino termini. N, Amino terminus; C, carboxy terminus. The shaded area is the region of the genome in which internal initiation of synthesis of proteins Q, R, S, T, Y, and Z may occur (see text).

of processing of P1-1a or of premature termination of protein synthesis. The origin of this small protein is unknown, although it has been postulated that it is the result of translation of one of the small open-reading frames in the poliovirus RNA sequence which precede the long open-reading frame encoding the polyprotein (5).

Previous studies of ribosome binding of poliovirus RNA have not resolved the enigma of the initiation of poliovirus protein synthesis in vitro. Classical ribosome binding studies conducted in reticulocyte lysate by this (A. J. Dorner, Ph.D. thesis, State University of New York at Stony Brook, Stony Brook, N.Y., 1983) and other (E. Ehrenfeld, University of Utah, Salt Lake City, personal communication) laboratories have failed to identify a unique initiation site sequence in vitro. Instead, ribosome binding to more than one sequence within the RNA was found (Dorner and Wimmer, unpublished data). More recently, electron microscopic evidence has been presented for the existence of three ribosome binding sites on the poliovirus RNA which map to widely separated regions of the genome (23). The accumulated evidence thus suggests that more than one initiation site could be utilized during poliovirus RNA translation in vitro.

In this paper, we examine the translation of poliovirus RNA in reticulocyte lysate. Analysis of the poliovirus-specific protein products by immunoprecipitation and *N*-formyl-[^{35}S]methionine labeling studies indicated that more than one initiation site was utilized in this cell-free system. In addition to the initiation site at the 5' end of the RNA, another strong initiation site(s) was identified within the 3'-proximal sequences. Utilization of this site resulted in the synthesis of a group of proteins derived from the P3 region of the polyprotein. Fragmentation of RNA cannot account for this phenomenon, and we concluded that internal initiation of protein synthesis was responsible for the complexity of the poliovirus-specific protein products observed after translation of poliovirus RNA in reticulocyte lysate. We further found that the addition of components of a HeLa cell extract to the reticulocyte lysate system reduced, if not completely abolished, this internal initiation of translation.

MATERIALS AND METHODS

Preparation of cell-free extracts. Rabbit reticulocyte lysate was prepared essentially as described previously (6, 14). A female New Zealand white rabbit was injected subcutaneously with 1 ml of 2.5% phenylhydrazine hydrochloride (0.14 M NaCl, pH 7.0) for 5 consecutive days. Two days after the last injection, the rabbit was exsanguinated by cardiac puncture. Blood cells were pelleted by centrifugation at 2,000 rpm for 12 min in an International centrifuge (IEC, Needham Heights, Mass.) and washed twice with ice-cold wash buffer (0.14 M NaCl, 50 mM KCl, 5 mM $Mg(OAc)_2$). Cells were lysed on ice for 10 min with an equal volume of cold distilled water. Cell debris was removed by centrifugation at 12,000 rpm for 15 min in a Sorvall SS34 rotor. The supernatant was stored in small portions at −70°C. Reticulocyte lysate was also purchased commercially (Bethesda Research Laboratories, Inc.).

The poliovirus-infected HeLa cell extract was prepared essentially as described previously (3). A total of 5×10^8 HeLa S3 cells were infected with poliovirus type 1 (Mahoney strain) at a multiplicity of 100 PFU per cell. Four hours after infection, the cells were harvested and washed twice with cold Earle salts solution. An equal volume of cold lysis buffer (10 mM KCl, 1.3 mM $Mg(OAc)_2$, 2.5 mM dithiothreitol, 10 mM HEPES[*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]–KOH, pH 7.4) was added, and the cells were left on ice for 20 min. The cells were then disrupted by 10 strokes in a Dounce homogenizer. Cell debris and nuclei were removed by centrifugation at 3,000 rpm for 5 min in an International centrifuge. Mitochrondria were removed from the supernatant by centrifugation at 10,000 rpm for 15 min in a Sorvall SS34 rotor. The supernatant was adjusted to 20% glycerol and stored in small portions at −70°C.

Cell-free protein synthesis. The conditions for in vitro protein synthesis were as described previously (7, 11). Reticulocyte lysate and HeLa cell extract were made mRNA dependent by treatment with micrococcal nuclease (14). Conditions for protein synthesis in reticulocyte lysate, excluding the contribution of the lysate, were as follows: 10 μl of lysate in a 25-μl reaction contained 20 mM HEPES-KOH, pH 7.6, 80 to 120 mM K(OAc), 0.1 mM $Mg(OAc)_2$, 0.5 mM spermidine hydrochloride, 4.5 mM dithiothreitol, 20 mM creatine phosphate, 0.1 mg of creatine phosphokinase per ml, 0.08 mM amino acids minus methionine, and 0.05 mg of rabbit liver tRNA per ml. Poliovirion RNA was added to a final concentration of 40 μg/ml. A total of 10 μCi of [^{35}S]methionine (Amersham Corp.) was usually added to each 25-μl reaction. Standard incubation conditions were 2 h at 30°C. When supplemented with micrococcal nuclease-treated, poliovirus-infected HeLa cell extract, the reaction volume was increased to 30 μl by the addition of 5 μl of HeLa cell extract. Conditions for the poliovirus-infected HeLa cell extract were the same as those for the reticulocyte lysate, except 100 mM K(OAc) and 0.4 mM $Mg(OAc)_2$ were used and 1 mM ATP and 0.2 mM GTP were included in the translation mix. For *N*-formyl-methionine labeling in the reticulocyte lysate, 1 μCi of *N*-formyl-[^{35}S]methionine-labeled $tRNA_f^{Met}$ (New England Nuclear Corp.) was added to a 25-μl reaction and incubated for 40 min at 30°C. Other changes of standard conditions are indicated in the figure legends.

Immunoprecipitation of translation products. Immunoprecipitations were carried out as described previously (27). Briefly, 10 μl of a reticulocyte lysate translation reaction was boiled for 2 min in an equal volume of gel sample buffer (62 mM Tris hydrochloride [pH 6.8], 2% sodium dodecyl sulfate [SDS], 0.7 M mercaptoethanol, 10% glycerol) and diluted with 280 μl of ice-cold TENN buffer (50 mM Tris hydrochloride [pH 8.0], 5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40). Antiserum was added, and the mixture was kept on ice for 12 h. Activated *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring) were added and kept on ice for an additional 15 min. The mixture was then pelleted, and the bacterium-antibody pellet was washed three times with SNNTE buffer (5% sucrose, 1% Nonidet P-40, 0.5 M NaCl, 50 mM Tris hydrochloride [pH 7.4], 5 mM EDTA) and once with NTE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA, 50 mM NaCl). The final pellet was resuspended in gel sample buffer containing 0.005% bromophenol blue and boiled for 2 min. The mixture was pelleted, and the supernatant containing immunoprecipitated proteins was analyzed by gel electrophoresis. Preparation of rabbit anti-VP4 serum was carried out as described previously (9). Antisera directed against poliovirus nonstructural proteins were prepared as described previously (11, 27).

Polyacrylamide gel electrophoresis. Proteins were analyzed on two different gel systems: 7.5 or 12.5% polyacrylamide gels containing 0.1% SDS (21) and 8% polyacrylamide gels containing 0.38 M Tris acetate, pH 8.3 (31).

Preparation of poliovirion RNA. Suspension cultures of HeLa S3 cells (5×10^6 cells per ml) were infected with poliovirus type 1 (Mahoney) at a multiplicity of 50 PFU per cell. Cells were harvested at 7 h postinfection and lysed by three rounds of freezing and thawing in reticulocyte standard buffer containing Mg^{2+} (0.01 M NaCl, 0.01 M Tris hydrochloride [pH 7.4], 1.5 mM $MgCl_2$), and the cell nuclei were pelleted. The supernatant containing poliovirions was made 2 mM in EDTA and 0.1% in SDS, and the virus was pelleted by centrifugation at 80,000 × *g* for 3 h. Pelleted virus was dissolved in 0.1 buffer (0.1 M NaCl, 0.01 M Tris hydrochloride [pH 7.4], 1 mM EDTA) containing 0.5% SDS and applied to a 15 to 30% sucrose gradient in 0.1 buffer containing SDS. Centrifugation was carried out at 16,000 × *g* for 16 h. Gradient-purified virus was extracted with phenol-chloroform (1:1, vol/vol), and the poliovirion RNA was precipitated with ethanol. RNA was applied to a 15 to 30% sucrose gradient in 0.1 buffer containing SDS and centrifuged at 200,000 × *g* for 4.75 h as the final purification step.

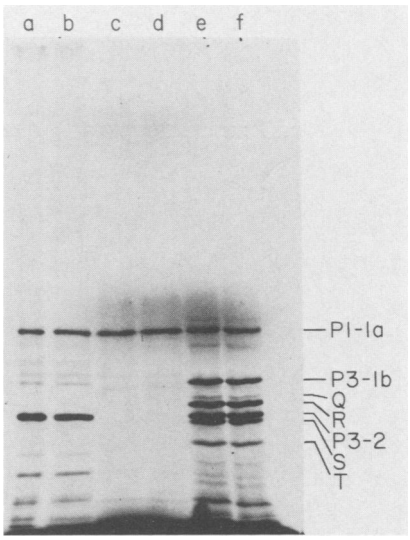

FIG. 2. Polyacrylamide gel electrophoresis of high-molecular-weight poliovirus-specific proteins synthesized in vitro. An autoradiogram of an 8% polyacrylamide gel of [^{35}S]methionine-labeled proteins is shown. Lanes a and b, lysate of poliovirus-infected HeLa cells labeled in vivo with [^{35}S]methionine; lanes c and d, micrococcal nuclease-treated, poliovirus-infected HeLa cell extract supplemented with poliovirion RNA; lanes e and f, micrococcal nuclease-treated reticulocyte lysate supplemented with poliovirion RNA. Note that the lower portion of this autoradiogram has been cut off owing to extensive blackening.

RESULTS

Electrophoretic analysis of high-molecular-weight translation products. Poliovirion RNA was translated in two cell-free protein synthesizing systems. An extract of poliovirus-infected HeLa cells can utilize the endogenous poliovirus RNA present in preformed viral polyribosomes to produce a pattern of proteins essentially identical to that observed in vivo or can be made messenger dependent by micrococcal nuclease treatment. This system contains the cellular translational machinery which has been modified by the poliovirus infection, resulting in shut-off of host cell protein synthesis (3). The micrococcal nuclease-treated reticulocyte lysate also translates poliovirion RNA, and both systems are active in proteolytic processing of the protein products. However, in our experiment, reticulocyte lysates or HeLa cell extracts prepared at different times and used by different investigators varied in the efficiency of poliovirus RNA translation and in the amount of protein processing. Each independent preparation was optimized for maximum incorporation of [^{35}S]methionine, and thus different translation experiments may have slightly different reaction conditions.

The reticulocyte lysate and HeLa cell extract exhibited similar but not identical patterns of protein products. An examination of the high-molecular-weight proteins synthe-

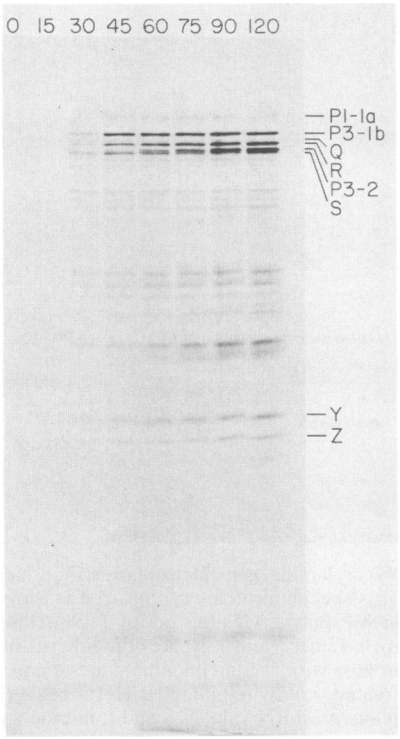

FIG. 3. Time course of protein synthesis in reticulocyte lysate programmed with poliovirus RNA. An autoradiogram of a 12.5% polyacrylamide gel of [^{35}S]methionine-labeled proteins synthesized at the indicated times (in minutes) after the addition of poliovirion RNA is shown. For conditions, see text.

sized in vitro by electrophoresis on an 8% polyacrylamide gel revealed major differences between the two systems. P1-1a, mapped at the initiation site of protein synthesis, was the predominant high-molecular-weight translation product in this preparation of messenger-dependent HeLa cell extract (Fig. 2, lanes c and d). Other preparations of HeLa cell extract exhibited a broader range of poliovirus-specific proteins. In addition to P1-1a, the products synthesized in the reticulocyte lysate included proteins which comigrated with P3-1b and P3-2, as well as several other major proteins (Q, R, S, and T) with estimated molecular weights of 78,000 to 68,000 (Fig. 2, lanes e and f). This complex band pattern was very reproducible, was not significantly altered by changes in the final concentration of added K^+ between 50 and 120 mM, and was not influenced by the use of KCl or potassium acetate. Of the abnormal proteins, R and S, which migrated slightly slower and slightly faster, respectively, than P3-2 (Fig. 2, lanes e and f), were the most abundant, whereas Q was always found in the lowest yield. These proteins did not have any in vivo counterparts (Fig. 2, lanes a and b) and were not synthesized in the micrococcal nuclease-treated, poliovirus-infected HeLa cell extract translating exogenous poliovirion RNA (Fig. 2, lanes c and d) or in the untreated extract translating endogenous poliovirus RNA (data not shown).

The time course of appearance of these products in the reticulocyte lysate was examined. For poliovirus RNA, typical lysates at an incubation temperature of 30°C would require 45 to 50 min for translation of the whole genome at a K^+ concentration of 60 mM and 33 to 36 min at a K^+ concentration of 100 mM (R. J. Jackson, unpublished data). These estimates suggest that the synthesis of the entire P1 segment should require up to 20 min at the lower K^+ concentration and about 15 min at the higher concentration. In an experiment in which the translation of poliovirus RNA proceeded relatively slowly, presumably because of the suboptimal K^+ concentration (80 mM) used, two small polypeptides (Y and Z) were the first products that could be detected (Fig. 3, 15 min). Significantly, P3-1b and the abnormal products Q, R, S, and T made their appearance by 30 min, before the appearance of P1-1a, which was first detected in significant amounts at the 45-min time point (Fig. 3). P3-2, a cleavage product from the P3 region encoded by the 3′-proximal RNA sequences, could be detected as a major band at the 60-min time point and increased during the duration of the time course.

***N*-Formyl-[^{35}S]methionine-labeled proteins.** The early synthesis of the high-molecular-weight proteins at the same time or before the appearance of P1-1a prompted an examination of their relationship to protein synthesis initiation in the reticulocyte lysate. *N*-Formyl-[^{35}S]methionine-labeled translation products from the reticulocyte lysate were analyzed on a 12.5% polyacrylamide gel. Several proteins carried the *N*-formyl-[^{35}S]methionine label after 40 min of protein synthesis (Fig. 4, lane b). Predominant among these proteins were P1-1a and proteins Q, S, T, Y, and Z. (We are not certain which of the two proteins, Q or R, is actually labeled and have assigned Q solely on the basis of its migration in the polyacrylamide gel. This uncertainty does not influence the conclusions drawn from the labeling ex-

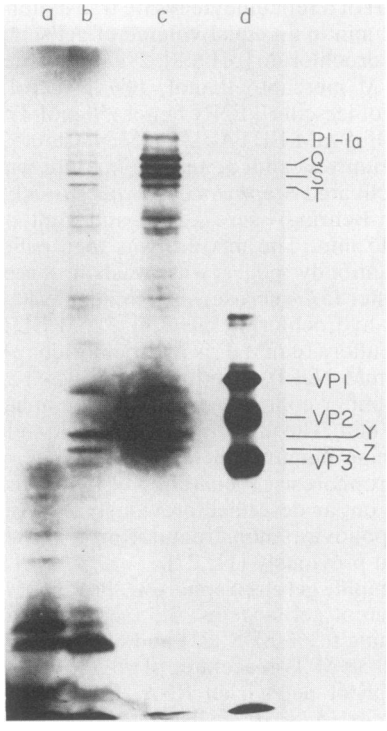

FIG. 4. Polyacrylamide gel electrophoresis of poliovirus-specific proteins labeled with *N*-formyl-[^{35}S]methionine in the reticulocyte lysate. An autoradiogram of 12.5% polyacrylamide gel subjected to fluorography is shown. Lane a, *N*-formyl-[^{35}S]methionine-labeled proteins synthesized in the absence of RNA; lane b, *N*-formyl-[^{35}S]methionine-labeled proteins synthesized in the presence of poliovirion RNA; lane c, [^{3}H]alanine-labeled proteins synthesized in the presence of poliovirion RNA; lane d, [^{35}S]methionine-labeled capsid proteins.

periment.) Of the high-molecular-weight proteins in the range of 68,000 to 85,000, S was always the most heavily labeled, followed by T and then Q. Labeling of P3-2 and R was never observed, whereas very weak labeling of a protein which had the same, or closely similar, electrophoretic mobility as P3-1b was a consistent feature. In the absence of poliovirus RNA, only low-molecular-weight labeled products were seen (Fig. 4, lane a); these were probably *N*-formyl-[^{35}S]methionine tRNA itself and labeled peptidyl-tRNA resulting from the translation of 5′-proximal fragments of globin mRNA in the nuclease-treated lysate. If the label used during in vitro protein synthesis was [^{3}H]alanine, the same group of initiation proteins emerged in addition to authentic poliovirus polypeptides that do not correspond to initiation proteins (Fig. 4, lane c).

These data indicate that, in addition to the expected labeling of the capsid precursor P1-1a, proteins Q, S, T, Y, and Z also carried amino termini encoded at an initiation site of protein synthesis. This was an unusual and unexpected result, because the available evidence suggests that poliovirus RNA has only one site of initiation of translation and that translation starts with the synthesis of P1-1a (5, 16, 30). To determine whether the other *f-met*-labeled proteins were the result of premature termination of protein synthesis initiated at the 5′ end of the poliovirus coding sequence or were due to aberrant processing of P1-1a, we identified the origin of the high-molecular-weight proteins by immunoprecipitation.

Immunoprecipitation of poliovirus-specific translation products. Immunoprecipitation of the proteins synthesized in the reticulocyte lysate enabled the identification of the region of the poliovirus polyprotein from which the observed translation products were derived. Translation reactions were immunoprecipitated with anti-VP4, anti-P3-2, or anti-P2-X serum after 35 or 220 min of protein synthesis (Fig. 5). The 35-min time point was chosen because calibration of the rate of translation in this particular lysate showed that, under these conditions, 35 min was insufficient time for complete translation of the whole open-reading frame, and therefore any high-molecular-weight products from the P3 region present at this time must have arisen from internal initiations. Anti-VP4 serum (9) was diagnostic for proteins carrying the amino-terminal sequences of the P1 region and immunoprecipitated P1-1a at both early and late times. After 220 min of protein synthesis, a small amount of VP0 could be immunoprecipitated by anti-VP4 serum. VP0 is derived from P1-1a by proteolytic processing. No VP4 could be observed, which was not surprising, because the cleavage of VP0 to produce VP4 and VP2 occurs only upon virion maturation. Anti-P2-X serum (11) identified proteins encoded within the middle of the genome and immunoprecipitated several major proteins. These proteins corresponded to P2-3b, P2-5b, and P2-X. P2-X increased with time, whereas P2-3b and P2-5b decreased, consistent with their precursor-product relationship.

P3-1b, P3-2, and proteins Q, R, S, T, Y, and Z were all immunoprecipitated by anti-P3-2 serum (27). Of these proteins, P3-2 was present in a high quantity only at 220 min, coincident with a decrease in the intensity of the P3-1b band, consistent with their precursor-product relationship. All of the other proteins that could be precipitated by anti-P3-2 serum were present at early times and were relatively stable. No proteins were immunoprecipitated with preimmune serum (Fig. 5, lanes N). These immunoprecipitation data enabled the identification of the P3 region of the polyprotein as the origin of those anomolous proteins identified by *N*-formyl-methionine labeling and time course studies.

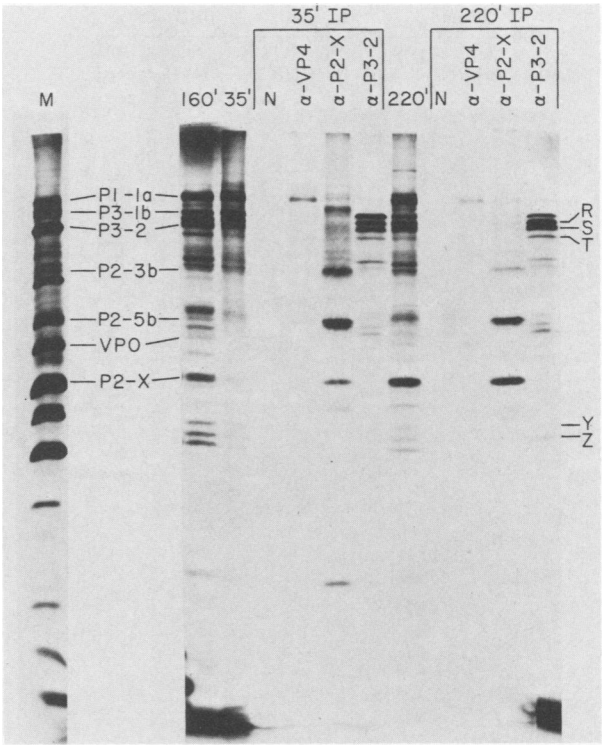

FIG. 5. Immunoprecipitation of poliovirus-specific proteins synthesized in reticulocyte lysate. An autoradiogram of a 12.5% polyacrylamide gel of immunoprecipitated [^{35}S]methionine-labeled proteins is shown. The reaction mixture (25 μl) contained 20 μl of reticulocyte lysate, 55 mM KCl, 0.27 mM $MgCl_2$, 10 mM creatine phosphate, 50 μg of creatine kinase per ml, 100 μM unlabeled amino acids, 60 μg of calf liver tRNA per ml, 10 μg of poliovirus RNA per ml, 7 mM dithiothreitol, and 400 μCi of [^{35}S]methionine per ml. Translation products were examined after 35 min (lanes 35′ IP) or 220 min (lanes 220′ IP) of protein synthesis at 30°C. Lanes N, immunoprecipitation with preimmune serum; lanes α-VP4, immunoprecipitation with anti-VP4 serum; lanes α-P2-X, immunoprecipitated with anti-P2-X serum; lanes α-P3-2, immunoprecipitated with anti-P3-2 serum; lanes 35′, 160′, and 220′, no immunoprecipitation of reactions after 35, 160, and 220 min of protein synthesis, respectively; lane M, lysate of poliovirus-infected HeLa cells labeled in vivo with [^{35}S]methionine.

These data were inconsistent with the utilization of a single initiation site of protein synthesis in vitro. On the contrary, they suggest that an internal initiation site(s) located within the 3′-proximal sequences of the poliovirus genome was active in the reticulocyte lysate in addition to the 5′-terminal initiation site at which the P1-1a encoding sequences are located. Alternatively, our results could be interpreted to suggest that high-molecular-weight poliovirus RNA was fragmented in the reticulocyte lysate and that such fragmentation produced new 5′ ends that were utilized for aberrant initiation of translation. The following experiment argues strongly against this possibility.

Addition of HeLa cell extract to reticulocyte lysate. A striking observation was the absence of the aberrant P3 region proteins from the translation products of the poliovirus-infected HeLa cell extract or the poliovirus-specific proteins synthesized in vivo. It is possible that the internal initiation events in vitro were the result of a deficiency in the reticulocyte lysate of a factor(s) necessary for correct 5′-proximal initiation of poliovirus protein synthesis and that

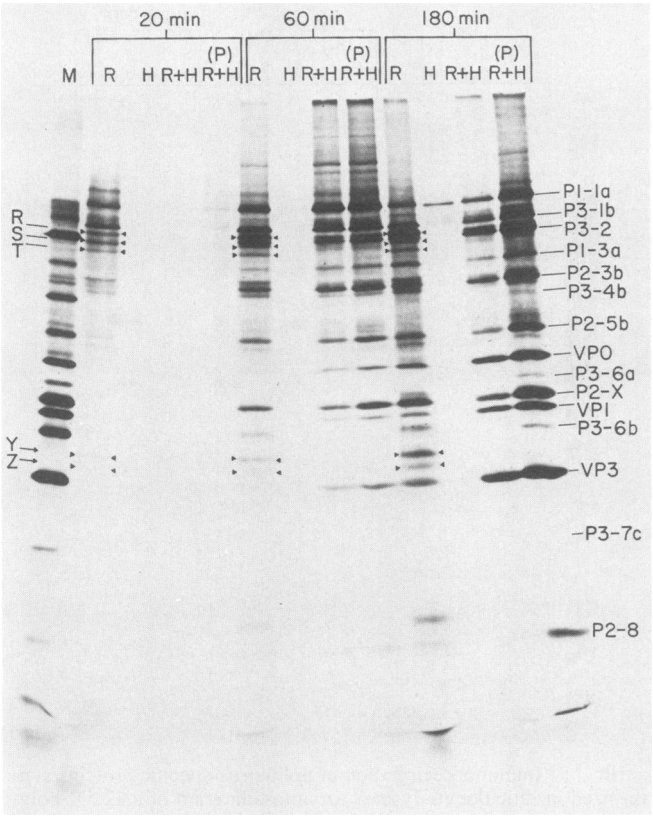

FIG. 6. Poliovirus polypeptides synthesized in rabbit reticulocyte lysate in vitro translation system in either the presence or the absence of infected HeLa cell extract. Micrococcal nuclease-treated rabbit reticulocyte lysate was purchased from Bethesda Research Laboratories. Protein synthesis mixtures (30 μl) contained 10 μl of the lysate and the following components: 40 mM KCl, 0.065 mg of calf liver tRNA per ml, 6 mM dithiothreitol, 86 mM KOAc, 25 mM HEPES-KOH (pH 7.5), 10 mM creatine phosphate, and 90 μM each of 19 unlabeled amino acids. Poliovirion RNA was added to 17 μg/ml, and [^{35}S]methionine was added to 500 μCi/ml. The micrococcal nuclease-treated, poliovirus-infected HeLa cell extract was prepared essentially as described by Celma and Ehrenfeld (3). The conditions for translation in rabbit reticulocyte lysate supplemented with infected HeLa cell extract were as described above, except the translation mixture also received 5 μl of HeLa cell extract. Cell-free protein synthesis in the micrococcal nuclease-treated, poliovirus-infected HeLa cell extract was carried out as described previously (4, 11). Poliovirus RNA was added to a final concentration of 22 mg/ml, and [^{35}S]methionine was added to 500 μCi/ml. Each translation reaction was incubated at 30°C for 20, 60, and 180 min. R, Translation of poliovirion RNA in rabbit reticulocyte lysate; H, translation of poliovirion RNA in micrococcal nuclease-treated, poliovirus-infected HeLa cell extract; R+H, poliovirus proteins synthesized in rabbit reticulocyte lysate supplemented with nuclease-treated, poliovirus-infected HeLa cell extract; (P) R+H, identical to lane R+H, except the translation mixture was preincubated at 30°C for 20 min before the addition of poliovirion RNA and [^{35}S]methionine. The aberrant polypeptides R, S, T, Y, and Z are indicated throughout the lanes with triangles.

this deficiency was absent from HeLa cell lysates. To examine this possibility, we supplemented the standard reticulocyte lysate reaction with micrococcal nuclease-treated, poliovirus-infected HeLa cell extract and analyzed the translation products by polyacrylamide gel electrophoresis.

The micrococcal nuclease-treated, poliovirus-infected HeLa cell extract synthesized predominantly P1-1a in response to poliovirus RNA (Fig. 6, lanes H), whereas proteins R, S, T, Y, and Z were among the translation products of the reticulocyte lysate at all three time points (Fig. 6, lanes R). The addition of HeLa cell extract altered the electrophoretic pattern of translation products observed in the reticulocyte lysate. Proteins R, S, T, Y, and Z were not synthesized in the reticulocyte lysate supplemented with HeLa cell extract (Fig. 6, lanes R + H). Preincubation of the reticulocyte lysate supplemented with HeLa cell extract for 20 min before the addition of RNA and radioactive label also resulted in the disappearance of these proteins (Fig. 6, lanes (P) R+H). Apparently, the addition of a component of the poliovirus-infected HeLa cell extract prevented the utilization of the internal initiation site(s) from which the anomolous P3 region proteins are synthesized. This effect is not diminished by a 20-min preincubation period, indicating that the factor responsible for the alteration in translation is relatively stable. Similar results have been obtained by the addition of uninfected HeLa cell extract (data not shown). This effect is not the result of a change in Mg^{2+} or K^{+} concentrations (data not shown).

DISCUSSION

Our analysis of poliovirus RNA translation in reticulocyte lysate has revealed a complex pattern of protein synthesis inconsistent with the utilization of a single initiation site. Four major high-molecular-weight (78,000 to 68,000) proteins and two proteins of ca. 28,000 in molecular weight which do not have counterparts in vivo were synthesized. These proteins appeared early in a time course of protein synthesis concomitantly with or before the appearance of P1-1a. All but one of these proteins (Q, S, T, Y, and Z) were labeled with *N*-formyl-[^{35}S]methionine, a marker that identifies them as initiation proteins.

Immunoprecipitation by anti-P3-2 serum but not by anti-VP4 or anti-P2-X serum showed that these proteins were encoded within the 3′-terminal sequences of poliovirus RNA and were translated within the long open-reading frame which encodes the P3 region of the polyprotein. These data exclude the possibility that these proteins were premature termination products of protein synthesis initiated at the 5′ end of the RNA or were the result of aberrant processing of P1-1a. Such events would produce proteins carrying the amino-terminal sequences of P1-1a which would have been identified by immunoprecipitation with anti-VP4 serum.

The data presented here indicated that more than a single initiation site is active in reticulocyte lysate. In vivo, the synthesis of the polyprotein, with P1-1a at its amino terminus, is initiated 743 nucleotides from the 5′ end of the RNA (5). Indeed, P1-1a is labeled with *N*-formyl-methionine during protein synthesis in the reticulocyte lysate, indicating that proper initiation in the 5′-terminal region of the RNA is occurring. However, other strong initiation sites have been located within the 3′ end sequences of the RNA that are utilized to produce proteins derived from the P3 region of the polyprotein. These proteins were not detected in a poliovirus-infected HeLa cell extract.

Fragmentation of mRNA may expose functional initiation sites which are inactive when the sequences are located internally on intact RNA (19). The following considerations argue against the possibility that RNA degradation in vitro is responsible for the initiation events reported here. The synthesis of proteins Q, R, S, T, Y, and Z could not be detected when reticulocyte lysate was supplemented with HeLa cell extract. This result is not consistent with initiation of protein synthesis on fragmented RNA unless the HeLa

cell extract contains inhibitors of RNA degradation not present in reticulocyte lysate. We know of no evidence to suggest that the HeLa cell extract contains such specific nuclease inhibitor(s). In fact, a previous study of the stability of poliovirus RNA in vitro has shown that degradation of RNA occurs in HeLa cell extract as well as in reticulocyte lysate (8), and RNA is less rapidly degraded in the reticulocyte lysate system than in HeLa cell extract. Yet the internal initiation of protein synthesis in vitro which produced the anomolous P3 region proteins was not observed in mixing experiments or in HeLa cell extract. Finally, in vitro translation of poliovirus RNA in reticulocyte lysate in the presence of human placental RNase inhibitor produced all of the aberrant polypeptides (data not shown). We conclude that the phenomenon we observed represents genuine initiation within the 3'-proximal sequences of poliovirus RNA and not at the 5' terminus of a degradation product.

The absence of these P3 region proteins from the poliovirus-specific translation product in HeLa cell extract or the proteins found in infected cells suggests that this internal initiation event may reflect some unusual characteristic of reticulocyte lysate. In support of this conclusion is the observation that the addition of HeLa cell extract to reticulocyte lysate resulted in the disappearance of the aberrant P3 region proteins. Apparently, the addition of one or several components of the HeLa cell extract can direct 5'-terminal initiation to the exclusion of this internal initiation event. It has previously been reported that the addition of a ribosomal salt wash from HeLa cells to reticulocyte lysate alters the electrophoretic pattern of translation products by decreasing the complexity of the pattern and increasing the number of proteins coelectrophoresing with authentic poliovirus-specific proteins in a fashion very similar to that described here (2). We may now attribute this reported change to an alteration in initiation site activity.

An unusual pattern of protein labeling was observed by Koch et al. when poliovirus-infected cells were subjected to osmotic shock (18). It is possible that internal initiation is responsible for this phenomenon also.

It is possible that faithful translation of poliovirus RNA has an abnormally high requirement for one or more factors and that this requirement is not fulfilled in reticulocyte lysate. It has recently been suggested that poliovirus RNA needs more initiation factor eIF-4A for efficient initiation of translation than cellular mRNAs or other picornavirus RNAs (4). Whether the addition of eIF-4A or any other initiation factor to reticulocyte lysate extract will diminish the synthesis of the aberrant polypeptides is currently being determined (for a discussion of the initiation of translation in the reticulocyte lysate, see reference 13).

Knowledge of the complete poliovirus RNA nucleotide sequence allows speculation on the location of the initiation site(s) utilized for synthesis of these aberrant P3 region proteins. Because they were immunoprecipitated by anti-P3-2 serum but not by anti-P2-X serum, we concluded that they are encoded within the 3'-terminal sequences past N5106. N5106 is the nucleotide assignment for the cleavage site between the P2 and P3 regions of the polyprotein (see Fig. 1). The termination codon for the long open-reading frame is at N7371, and synthesis of a protein of the estimated size of the largest aberrant P3 region protein (78,000 MW) would require an initiation event to occur around N5300. AUG codons are located at N5290, N5346, N5466, and N5517 in the nucleotide sequence (17) and some or all of these could be involved in internal initiation. The region of the poliovirus genome that we have implicated in the internal initiation of protein synthesis is indicated in Fig. 1. Corroborating these considerations, a study of ribosome binding of poliovirus RNA in reticulocyte lysate located a putative ribosome binding site estimated to be 5,300 nucleotides from the 5' end of the RNA (23).

Protein Q synthesis may be initiated at the AUG codon at N5290. Protein R may be produced by amino-terminal processing of protein Q at one of the Gln-Gly cleavage sites in this region. Although protein R appeared early in the time course, it was not labeled with *N*-formyl-[^{35}S]methionine. Amino-terminal processing of protein Q would explain why protein Q was present in only small amounts relative to the other P3 region proteins and explain the absence of the *N*-formyl-[^{35}S]methionine label on protein R. Indeed, in some experiments, this small amount of Q was seen to disappear upon further incubation with cycloheximide (data not shown). Proteins S and T may be the products of initiation at the AUG codons at N5466 and N5517, respectively. Proteins Y and Z may be processing products or the results of premature termination of protein synthesis. Identification of the exact coding sequences awaits sequence or tryptic peptide analysis of these in vitro translation products.

P3-1b appeared at approximately the same time as proteins Q, R, S, and T and was usually detectable before P1-1a or any larger products. This observation indicates that, in the early stages of the translation reaction, P3-1b may originate from an internal initiation event. However, P3-1b was not labeled significantly with *N*-formyl-[^{35}S]methionine (see Fig. 4). A possible explanation for this observation is that the P3-1b observed at early times is produced by the cleavage of a precursor protein which spans the cleavage site between the P2 and P3 regions. Efficient removal of an amino-terminal portion (possibly only of methionine) from a precursor would result in the appearance of a polypeptide that would migrate like P3-1b. The initiation event for such a precursor must occur 5' of N5106, and possible initiation codons could be found at N5043 and N5088.

In light of these considerations, the results published by Shih et al. (28) should be reevaluated. These authors assumed that a polypeptide migrating in gels like P3-1b was indeed P3-1b and that it was the product of polyprotein processing already by 7.5 min in the time course of their translation in reticulocyte lysate. Shih et al. (28) concluded that, by 7.5 min, the ribosome had transversed the entire poliovirus genome, a process taking place three times faster than that required for translation of encephalomyocarditis virus RNA (another picornavirus mRNA). The low K^+ concentration (40 mM) used in their translation mixture makes it unlikely that polyprotein synthesis and processing could have occurred in such a short time. Moreover, P1-1a does not accumulate with the same kinetics as the putative P3-1b in these studies (28). We therefore suggest that the rapid appearance of a P3-1b-like protein observed by Shih et al. (28) is also the result of internal initiation of translation.

The mechanism that leads to the utilization of AUG codons several thousand nucleotides downstream from the 5' end to initiate translation is unknown. We speculate that the secondary structure of the RNA may play a role in ribosome binding to this region. A peak of adenosine-uridine-rich and guanosine-cytosine-poor secondary structure has been located within this region by computer analysis (A. Jacobson, State University of New York at Stony Brook, Stony Brook, personal communication). This type of relaxed secondary structure has also been noted at the 5'-terminal initiation site of polyprotein translation (5) and may facilitate ribosome binding. The initiation site that occurs

743 nucleotides downstream from the 5′ end of the RNA is preceded by eight AUG codons and numerous stop codons distributed over all three reading frames. The unusual arrangement of AUG codons of poliovirus mRNA (which is identical in nucleotide sequence with genome RNA) is difficult to reconcile with the ribosome scanning model (20). The possibility cannot be excluded that ribosome recognition of and ribosome binding to the 5′- and 3′-terminal sites occur by the same mechanism that is distinct from the scanning mechanism. Indeed, multiple internal initiations have been observed when the genome RNA of *E. coli* phage MS2 or R17 is translated in a cell-free eucaryotic extract (1), results which cannot be explained with the scanning mechanism. If internal initiation of translation occurs in vitro, the question arises as to whether the 3′-terminal initiation sites of poliovirus RNA are active in vivo. Such a property would be highly advantageous very early in the replicative cycle, when the virus needs proteinase and RNA polymerase, two nonstructural proteins encoded in the P3 region, for efficient protein processing and RNA replication. Conformational changes of the mRNA, possibly due to the binding of up to 35 ribosomes per RNA strand, may render the 3′-terminal sites inactive soon after the onset of viral translation.

ACKNOWLEDGMENTS

We thank Tsutomu Takegami and Carl W. Anderson for many stimulating discussions and Lydia F. Dorner for technical assistance.

This work was supported in part by Public Health Service grants AI 15122 and CA 28146 to E.W. from the National Institutes of Health and by a grant from the Medical Research Council of the United Kingdom to R.J.J. B.L.S. was a Postdoctoral Fellow of the National Institutes of Health (grant AI 05935).

LITERATURE CITED

1. **Atkins, J. F., J. A. Steitz, C. W. Anderson, and P. Model.** 1979. Binding of mammalian ribosomes to MS2 phage RNA reveals an overlapping gene encoding a lysis function. Cell **18**:247–256.
2. **Brown, B. A., and E. Ehrenfeld.** 1979. Translation of poliovirus RNA *in vitro*: changes in cleavage pattern and initiation sites by ribosomal salt wash. Virology **97**:396–405.
3. **Celma, M. L., and E. Ehrenfeld.** 1975. Translation of poliovirus RNA *in vitro*: detection of two different initiation sites. J. Mol. Biol. **98**:761–780.
4. **Daniels-McQueen, S., B. Morgan-Detjen, J. A. Grifo, W. C. Merrick, and R. E. Thach.** 1983. Unusual requirements for optimum translation of polioviral RNA *in vitro*. J. Biol. Chem. **258**:7195–7199.
5. **Dorner, A. J., L. F. Dorner, G. R. Larsen, E. Wimmer, and C. W. Anderson.** 1982. Identification of the initiation site of poliovirus polyprotein synthesis. J. Virol. **42**:1017–1028.
6. **Dorner, A. J., and B. Kemper.** 1978. Conversion of preproparathyroid hormone to proparathyroid hormone by dog pancreatic microsomes. Biochemistry **17**:5550–5555.
7. **Dorner, A. J., P. G. Rothberg, and E. Wimmer.** 1981. The fate of VPg during *in vitro* translation of poliovirus RNA. FEBS Lett. **132**:219–223.
8. **Ehrenfeld, E., and B. A. Brown.** 1981. Stability of poliovirus RNA in cell-free translation systems utilizing two initiation sites. J. Biol. Chem. **256**:2656–2661.
9. **Emini, E. A., A. J. Dorner, L. F. Dorner, B. A. Jameson, and E. Wimmer.** 1983. Identification of a poliovirus neutralization epitope through use of neutralizing antiserum raised against a purified viral structural protein. Virology **124**:144–151.
10. **Emini, E. A., M. Elzinga, and E. Wimmer.** 1982. Carboxy-terminal analysis of poliovirus proteins: termination of poliovirus RNA translation and location of unique poliovirus polyprotein cleavage sites. J. Virol. **42**:194–199.
11. **Hanecak, R., B. L. Semler, C. W. Anderson, and E. Wimmer.** 1982. Proteolytic processing of poliovirus polypeptides: antibodies to polypeptide P3-7c inhibit cleavage at glutamine-glycine pairs. Proc. Natl. Acad. Sci. U.S.A. **79**:3973–3977.
12. **Humphries, S., F. Knauert, and E. Ehrenfeld.** 1979. Capsid protein precursor is one of two initiated products of translation of poliovirus RNA *in vitro*. J. Virol. **30**:481–488.
13. **Jackson, R. J.** 1982. The control of initiation of protein synthesis in reticulocyte lysates, p. 362–418. *In* R. Perez-Bercoff (ed.), Protein biosynthesis in eukaryotes. Plenum Publishing Corp., New York.
14. **Jackson, R. J., and T. Hunt.** 1983. Protein synthesis in rabbit reticulocyte lysates. Methods Enzymol. **96**:50–74.
15. **Jacobson, M. F., J. Asso, and D. Baltimore.** 1970. Further evidence on the formation of poliovirus proteins. J. Mol. Biol. **49**:657–669.
16. **Jacobson, M. F., and D. Baltimore.** 1968. Polypeptide cleavages in the formation of poliovirus proteins. Proc. Natl. Acad. Sci. U.S.A. **61**:77–84.
17. **Kitamura, N., B. L. Semler, P. G. Rothberg, G. R. Larsen, C. J. Adler, A. J. Dorner, E. A. Emini, R. Hanecak, J. J. Lee, S. van der Werf, C. W. Anderson, and E. Wimmer.** 1981. Primary structure, gene organization, and polypeptide expression of poliovirus RNA. Nature (London) **291**:547–553.
18. **Koch, G., F. Koch, J. A. Billelo, E. Hiller, C. Scharli, G. Warnecke, and C. Weber.** 1982. Biosynthesis, modification and processing of viral polyproteins, p. 275–309. *In* R. Perez-Bercoff (ed.), Protein biosynthesis in eukaryotes. Plenum Publishing Corp., New York.
19. **Kozak, M.** 1980. Binding of wheat germ ribosomes to fragmented viral mRNA. J. Virol. **35**:748–756.
20. **Kozak, M.** 1983. Comparison of initiation of protein synthesis in prokaryotes, eukaryotes, and organelles. Microbiol. Rev. **47**:1–45.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
22. **Larsen, G. R., C. W. Anderson, A. J. Dorner, B. L. Semler, and E. Wimmer.** 1982. Cleavage sites within the poliovirus capsid protein precursors. J. Virol. **41**:340–344.
23. **McClain, K., M. Stewart, M. Sullivan, and J. V. Maizel.** 1981. Ribosomal binding sites on poliovirus RNA. Virology **113**:150–167.
24. **Rekosh, D.** 1972. Gene order of the poliovirus capsid proteins. J. Virol. **9**:479–487.
25. **Semler, B. L., C. W. Anderson, N. Kitamura, P. G. Rothberg, W. L. Wishart, and E. Wimmer.** 1981. Poliovirus replication proteins: RNA sequence encoding P3-1b and the sites of proteolytic processing. Proc. Natl. Acad. Sci. U.S.A. **78**:3464–3468.
26. **Semler, B. L., R. Hanecak, C. W. Anderson, and E. Wimmer.** 1981. Cleavage sites in the polypeptide precursors of poliovirus protein P2-X. Virology **114**:589–594.
27. **Semler, B. L., R. Hanecak, L. F. Dorner, C. W. Anderson, and E. Wimmer.** 1983. Poliovirus RNA synthesis *in vitro*: structural elements and antibody inhibition. Virology **126**:624–635.
28. **Shih, D. S., C. T. Shih, O. Kew, M. Pallansch, R. Rueckert, and P. Kaesberg.** 1978. Cell-free synthesis and processing of the proteins of poliovirus. Proc. Natl. Acad. Sci. U.S.A. **75**:5807–5811.
29. **Summers, D. F., and J. V. Maizel.** 1971. Determination of the gene sequence of poliovirus with pactamycin. Proc. Natl. Acad. Sci. U.S.A. **68**:2852–2856.
30. **Taber, R., D. Rekosh, and D. Baltimore.** 1971. Effect of pactamycin on synthesis of poliovirus proteins: a method for genetic mapping. J. Virol. **8**:395–401.
31. **Tegtmeyer, P., and B. Anderson.** 1981. Partial purification of SV40 A protein and a related cellular protein from permissive cells. Virology **115**:67–74.
32. **Villa-Komaroff, L., N. Guttman, D. Baltimore, and H. F. Lodish.** 1975. Complete translation of poliovirus RNA in a eukaryotic cell-free system. Proc. Natl. Acad. Sci. U.S.A. **72**:4157–4161.
33. **Warner, J., M. J. Madden, and J. E. Darnell.** 1963. The interaction of poliovirus RNA with *Escherichia coli* ribosomes. Virology **19**:393–399.